

Rationale

After recombination the minicircle-DNA should contain only the target sequence (antigen or therapeutic gene) including necessary regulatory elements for its expression (minimalistic transcription unit), the ligand sequences for the purification process (*lacO_r*-sites) and the ressite (the product of the recombination at the res1 and res2 sites). In order to create a minicircle with a minimalistic transcription unit, the res1 site on plasmid pHCNparA has been rearranged. Furthermore a multiple cloning site for the integration of respective target sequences was inserted. The resulting plasmid pRBPS1 can now be used for the insertion of any target sequence of interest to create the corresponding minicircle DNA. To demonstrate the almost complete recombination of the restructured plasmid the eukaryotic luciferase gene expression cassette was cloned in said multiple cloning site of pRBPS1 resulting in plasmid pRBPS1-luc-CMV.

Fig. 1: Construction of a universally usable origin plasmid pRBPS1:

An overview of the three-step cloning strategy is schematically presented in Fig. 1. Briefly, the resolution site 1 in plasmid pHCNparA had to be removed at first (Fig. 1A). Plasmid pHCNparA was digested with restriction endonucleases *Hind*III and *Pst*I. The linearized vector lacking res1 was isolated, the sticky ends were blunt ended with Klenow fragment and ligated. Thus, the *Hind*III and *Pst*I sites were destroyed in the resulting vector pHCN-res2. The vector pHCN-res2 carries a unique *Nsi*I site right upstream of the *lacOs* sites, which are needed for the specific binding of the minicircle DNA. A MCS, containing a variety of unique restriction sites were generated by annealing two oligonucleotides carrying the respective sequences. The MCS is shown in Fig. 1A. The MCS sequence was cloned in the *Nsi*I site of pHCN-res2 resulting in the plasmid pHCNmcs (Fig. 5B). The second resolution site was subsequently introduced in the newly generated *Hind*III and *Pst*I sites resulting in the universally useable origin plasmid pRBPS1 (Recombination Based Plasmid Separation) (Fig. 1C). Following features characterize this plasmid: (i) the *res1-lacOs-mcs-res2* cassette of pRBPS1 can now be cut out and placed elsewhere with the *Not*I and *Hind*III sites and (ii) the chosen target gene and its expression unit can be inserted into the MCS, which contains recognition sites for a broad variety of single cutters, between the two resolution sites in the vicinity of the *lacOs* sequence. MB1, origin of replication from the high-copy-number plasmid pUC19; bla, ampicillin resistance gene; P_{BAD}, arabinose-inducible promoter; araC, repressor/inducer gene of the P_{BAD} promoter; Os, *lac* operator sites with a tenfold higher binding affinity for the Lac repressor than the wild-type operator sites; spacer, unrelated spacer sequence derived from the archeal phage ϕ CH1; parA, parA resolvase gene; res1, res2, resolution sites; ParA recognition sequences; 5S1T2, parts of the 5S rRNA and the ribosomal *rrnB* transcription-terminators T1 and T2 of *E. coli*.

Fig. 2: Plasmid map of pRBPS1-luc and restriction analysis of the recombination products (minicircle and miniplasmid) after recombination.

- (A) Plasmid map of plasmid pRBPS1-luc. Plasmid pCMV-luc was digested with *Sph*I and *Afe*I (creates blunt ends). The resulting fragment carrying the CMV promoter - luciferase gene (*luc*) - polyadenylation site (poly A) cassette was inserted into the corresponding *Sph*I and the blunt-ended *Sma*I site in the multiple cloning site of plasmid pRBPS1. The resulting plasmid pRBPS1-luc-CMV encodes a minimal eukaryotic expression cassette (CMV-luc-polyA) and the *lacO*s sites flanked by two resolution sites (minicircle sequence, MC) as well as the miniplasmid (MP) sequences (parA resolvase under expression control of the araBAD expression system, antibiotic resistance gene, origin of replication). CMV; major immediate early enhancer containing promoter of the human cytomegalovirus; luc, luciferase gene; SV40 polyA, polyadenylation sequence of the SV40 virus necessary for transcriptional termination, MB1, origin of replication from the high-copy-number plasmid pUC19; bla, ampicillin resistance gene; P_{araD}, arabinose-inducible promoter; araC, repressor/inducer gene of the P_{araD} promoter; *lacO*s, *lac* operator sites with a tenfold higher binding affinity for the Lac repressor than the wild-type operator sites; spacer, unrelated spacer sequence derived from the archeal phage φCH1; parA, parA resolvase gene; res1, res2, resolution sites, ParA recognition sequences. Unique recognition sites for restriction endonucleases are marked with blue colour, restriction sites present on the plasmid pRBPS1-luc-CMV twice are indicated with red colour. The site where the blunt ended *Sma*I site from plasmid pRBPS1 was ligated together with the fragment derived blunt ended *Afe*I site is indicated (*Sma*I/*Afe*I).
- (B) *Bam*H I restriction analysis of parental plasmid pRBPS1-luc-CMV, minicircles and miniplasmids before and after recombination driven by the ParA resolvase in *E. coli* TB1 (pRBPS1-luc-CMV). DNA preparations (E.Z.N.A. Plasmid Miniprep Kit I, PEQLAB-Biotechnologie GmbH Erlangen, Germany) of *E. coli* TB1 (pRBPS1-luc-CMV): lane 2 and 3, before addition of 0.5% L-arabinose (overnight culture and immediately before induction). The parental plasmid pRBPS1-luc-CMV is divided into a 6868 bp (fragment 1 PP) and a 1525 bp fragment (fragment 2 PP) (see Fig. 2A); lane 4, 30 min after induction of the parA resolvase. The minicircles and miniplasmids are linearized having a length of 4040 bp (MC) and 4353 bp (MP), respectively. A small amount of unrecombined parental plasmid is still visible (fragment 1 PP); lane 5, 60 min after expression of the ParA resolvase. The minicircles and miniplasmids are linearized having a length of 4040 bp (MC) and 4353 bp (MP), respectively. No parental plasmid can be detected on the agarose gel; lane 1, 1 kb ladder, Invitrogen, Lofer, Austria.

(C) *Bam*H/*Scal* restriction analysis of parental plasmid pRBPS-luc-CMV, minicircles and miniplasmids before and after recombination driven by the *ParA* resolvase in *E. coli* TB1 (pRBPS-luc-CMV). DNA preparations (E.Z.N.A. Plasmid Miniprep Kit I, PEQLAB-Biotechnologie GmbH Erlangen, Germany) of *E. coli* TB1 (pRBPS-luc-CMV): lane 2, before addition of 0.5% L-arabinose. The parental plasmid pRBPS-luc-CMV is divided into 5295 bp (fragment 1 PP), a 1571 bp fragment (fragment 2 PP) and a 1525 bp fragment (fragment 3 PP) (see Fig. 2A); lane 3, 60 min after induction of the *parA* resolvase. The minicircle is linearized (linearized MC, 4040 bp) and the miniplasmid is divided into a 2782 bp (fragment 1 MP) and a 1571 bp fragment (fragment 2 MP), respectively. No parental plasmid can be detected on the agarose gel (fragment 1 PP); lane 1, 1 kb ladder, Invitrogen, Lofer, Austria.

Fig.1.

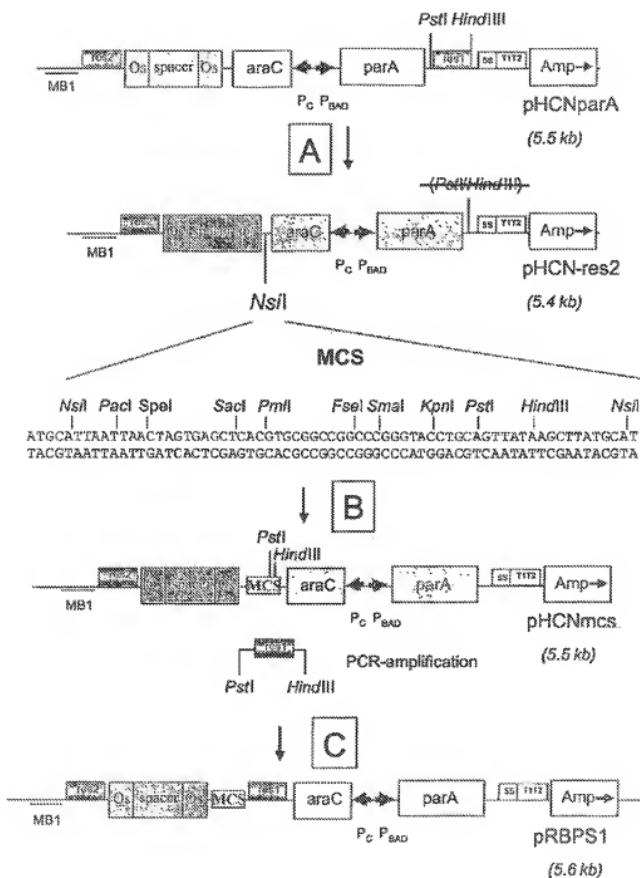


Fig. 2.

